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Reductase-catalyzed tetrahydrobiopterin regeneration alleviates the anti-competitive inhibition of tyrosine hydroxylation by 7,8-dihydrobiopterin†

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L-Tyrosine hydroxylation by tyrosine hydroxylase is a significant reaction for preparing many nutraceutical and pharmaceutical chemicals. Two major challenges in constructing these pathways in bacteria are the improvement of hydroxylase catalytic efficiency and the production of cofactor tetrahydrobiopterin (BH4). In this study, we analyzed the evolutionary relationships and conserved protein sequences between tyrosine hydroxylases from different species by PhyML and MAFFT. Finally, we selected 7 tyrosine hydroxylases and 6 sepiapterin reductases. Subsequently, the function of different groups was identified by a combined whole-cell catalyst, and a series of novel tyrosine hydroxylase/sepiapterin reductase (TH/SPR) synthesis systems were screened including tyrosine hydroxylase (from *Streptosporangium roseum* DSM 43021 and *Thermomonospora curvata* DSM 43183) and sepiapterin reductase (from *Photobacterium damselae*, *Chlorobaculum thiosulfatiphilum* and *Xenorhabdus poinarii*), namely as SrTH/PdSPR, SrTH/CtSPR, SrTH/XpSPR and TcTH/PdSPR, which can synthesize L-Dopa by hydroxylating L-tyrosine in *Bacillus licheniformis*. Furthermore, we analyzed the characterization of SrTH by enzyme catalysis and demonstrated that 7,8-dihydrobiopterin (BH2) formed by BH4 autooxidation was an anticompetitive inhibitor on SrTH. Finally, pure dihydropteridine reductase from *Escherichia coli* (EcDHPR) was added to the solution, and L-Dopa could be continually synthesized after 3 h, which was improved by 86% at 6 h in the catalytic reaction by SrTH. This indicates that BH4 regeneration can alleviate the inhibition by BH2 during tyrosine hydroxylation. This study provides a good starting point and theoretical foundation for further modification to improve the catalytic efficiency of tyrosine hydroxylation by tyrosine hydroxylase.

Introduction

L-Tyrosine hydroxylation is the rate-limiting step in the central nervous system and adrenal gland, where its role is to catalyze the first step in the biosynthesis of neuroactive chemicals, including L-Dopa, the catecholamines dopamine (DA), noradrenaline (NA) and adrenaline in animals.[1,2] Simultaneously, L-tyrosine hydroxylation also plays an important role in preparing multiple nutraceutical and pharmaceutical chemicals, such as melanin-like biomaterials, plant alkaloids, betalain pigments and monolignols.[4–8] In animals, tyrosine hydroxylation is achieved by tyrosinase and tyrosine hydroxylase requiring tetrahydrobiopterin (BH4) as a native cofactor.[2,3] Microbial L-tyrosine hydroxylation is generally achieved by tyrosinase and 4-hydroxyphenylacetate-3-hydrolase (HpaBC).[11,13–18]

Tyrosinase is a widely distributed multicopper oxygenase in bacteria, fungi, plants, and animals that has bifunctional activity on monophenols and *o*-diphenols.[15–17] It is an internal monooxygenase requiring molecular O_2 and two electrons from substrate in the catalysis process. The oxidase reaction from *o*-diphenol to *o*-quinone is much more rapid than the oxygenation reaction from monophenol to *o*-diphenol.[17] L-Dopa can be oxidized to L-dopaquinone *via* the diphenolase (DP) activity of tyrosinase, eventually turning into melanin.[19] HpaBC is a native enzyme complex in *E. coli* that is able to hydroxylate multiple substrates, such as 4-hydroxyphenylacetic acid, tyrosine, *p*-coumaric acid and 4-tyrosol, with NADH as a native

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cofactor for *hpaC* reducing FAD to FADH2 which is used for substrate hydroxylation by *hpaB*.[9–14] HpaBC was used to synthesize L-Dopa from glucose in previous study.[11,13,14] However, a large amount of melanin would be formed and a lot of tyrosine remained in the fermentation broth, which causes loss of carbon flux to L-Dopa and its downstream high-value products, such as hydroxytyrosol, plant alkaloids, betalain pigments and monolignols.[4–8,25] It is worth noting that tyrosine hydroxylase does not have diphenolase (DP) activity. In the previous study, Jay D. Keasling and Taek Soon Lee used TH from mouse for the production of L-Dopa and hydroxytyrosol with MH4 as cofactor in *E. coli*.[25] The conserved amino acid positions and substrate binding site of tyrosine hydroxylase from animals have been elaborated.[21,35,39] There is no report on tyrosine hydroxylase from microorganisms. The specific enzyme activity of tyrosine hydroxylase from human and mouse is 1017 nmol min^{-1} (mg of protein)$^{-1}$ at 25 °C and 2.78 nmol min^{-1} (mg of protein)$^{-1}$ at 30 °C in 10 mM NaHepes buffer (pH 7.0).[20,40] However, the specific enzyme activity of TH from animals at 37 °C reduced to 15.4% of that at 30 °C, which indicates that tyrosine hydroxylase from animals is sensitive to high temperature.[40] Additionally, tyrosine hydroxylase from animals can be inhibited by many chemicals, such as L-tyrosine (over 100 μM), oxygen (higher than 4.8%), 4-amino-BH4, 5-methyl-BH4 and 8-M-6,7-DMPH4.[20,21]

The hydroxylation of L-tyrosine by tyrosine hydroxylase requires the participation of the cofactor BH4, L-tyrosine and oxygen. Fortunately, BH4 can be successfully synthesized in *E. coli*, reaching 718 mg L^{-1} by AG14/(pSTV28GPS, pMW218guaBA) by using GCHI from *B. subtilis* which is also present in *B. licheniformis* and has lower K_m value than FolE from *E. coli*.[22,47,48] The titer of 5-hydroxytryptophan, also requiring BH4 as a cofactor, can reach up to 1.61 g L^{-1} in shaking flasks by *E. coli*, indicating that it is feasible to efficiently synthesize hydroxyl chemicals with BH4 as a cofactor.[3,23,24] However, this goal by tyrosine hydroxylase has not been achieved, although a new pathway producing L-DOPA by tyrosine hydroxylase has been demonstrated in the progress of synthesizing hydroxytyrosol in *E. coli*, which may be due to the poor catalytic efficiency at 37 °C of tyrosine hydroxylase from mouse.[25,40] Therefore, we tried to screen tyrosine hydroxylase/sepiapterin reductase (TH/SPR) synthesis systems from microorganisms.

In this study, we describe tyrosine hydroxylation by tyrosine hydroxylase from *Streptosporangium roseum* DSM 43021 (SrTH) in *B. licheniformis* and the characterization of SrTH during the catalytic process; that is, (1) screening of tyrosine hydroxylases and sepiapterin reductases from NCBI by computer-assisted sequence analysis, (2) functional identification of TH/SPR dual-enzyme synthesis systems by combined whole-cell catalyst, (3) inhibition analysis of dihydrobiopterin during crude enzyme reaction, (4) characterization of inhibition by BH2 on SrTH partially purified *via* ion exchange chromatography and (5) evaluating the effectiveness of pure dihydropteridine reductase from *E. coli* (EcDHPR) addition on the reaction catalyzed by crude SrTH.

Experimental section

Computer-assisted sequence analysis

The protein sequences in this study were obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments of protein sequences were performed with the program MAFFT (MAFFT-linsi) v7.471 (ref. 26) and visualized with ESPript 3.0 (ref. 27) to identify strictly conserved amino acid residues. The maximum-likelihood phylogenetic tree of tyrosine hydroxylase was created by PhyML v3.0 after selecting the best amino acid substitution model by ProtTest v3.4.2.[28]

Microorganisms and cultivation conditions

B. licheniformis CICIM B6902, stored in the Culture and Information Center of Industrial Microorganisms of China Universities (CICIM-CU), was used in this study, and the pMA5 plasmid was provided by Beinuo Life Science (Shanghai, China). *E. coli* DH5α (CICIM B0006) and *B. licheniformis* CICIM B6902 seed cultures were maintained in 150 mL flasks containing 15 mL of lysogeny broth (LB) at 37 °C and 200 rpm on a rotary shaker for 12 h. The following antibiotics were added as appropriate to the growth media: 30 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. For enzyme expression, cells were grown at 37 °C and 250 rpm in fermentation medium (pH 7.0) containing (per liter) lactose (30 g), yeast extract (24 g), peptone (12 g), $K_2HPO_4{\cdot}3H_2O$ (16.43 g) and KH_2PO_4 (2.3 g).

Construction of plasmids and recombinant strains

All the constructed strains and plasmids are listed in Table S1,† and all the primers for constructing plasmids are listed in Table S2.† The codon-optimized genes from different microorganisms were synthesized by Synbio Technologies (Suzhou, China). All synthesized genes were submitted to the NCBI GenBank database. The GenBank accession numbers and sequences of all genes are listed in Table S3.† The PCR products were digested with *Nde*I and *Bam*HI and cloned into *Nde*I–*Bam*HI-digested pMA5 after agarose gel purification. The ligation mixtures were first transformed into chemically competent *E. coli* DH5α cells. The positive recombinants were first selected by the phenotype showing ampicillin resistance and further confirmed by PCR.

Enzyme assays

The TH activity was assayed at 37 °C, using an incubation mixture containing 20 mM sodium phosphate buffer (pH 7.0), 50 mg L^{-1} L-tyrosine, 0.25 mmol L^{-1} BH4 and 0.25 μM Fe_2SO_4. The enzyme was preincubated for 10 min with 0.25 μM Fe_2SO_4 and 0.25 mmol L^{-1} BH4 before the reaction was started by addition of L-tyrosine. The reaction kept 3 min at 37 °C and 2 mmol L^{-1} sodium periodate was added into the solution. The mix kept at 25 °C in the dark for 10 min. All experiments were done at a final volume of 222 μL and 200 μL was taken into a 96-well plate for measurement at 475 nm.[50]

Hydroxylation of tyrosine by synergetic whole-cell catalysts

After verification by DNA sequencing, the plasmids harboring different genes were transferred by electroporation into *B. licheniformis*. The positive recombinants were first selected by the phenotype showing kanamycin resistance and further confirmed by PCR. *B. licheniformis* transformants were inoculated into 50 mL of fermentation medium supplied with 30 μg mL^{-1} kanamycin and 3% (v/v) seed cultures in 250 mL flasks and cultured at 37 °C and 250 rpm for 24 h. After fermentation, the OD_{600} was measured. The cells were collected by centrifugation at 4 °C and 12 000 rpm for 10 min, washed twice with 10 mmol L^{-1} sodium phosphate buffer (pH 7.0) and resuspended in the above buffer to an OD_{600} of 20. The two kinds of cells expressing TH or SPR were mixed 1 : 1 in a two-by-two combination. In the process, 2 g L^{-1} L-tyrosine was added, and the reaction was at 37 °C and 250 rpm for 24–36 h with 30 mL in 250 mL flasks. After combining whole-cell catalysts, the samples were centrifuged at 4 °C and 12 000 rpm for 20 min, and the supernatant was treated with 5% trichloroacetic acid. After 12 h at 4 °C, the samples were centrifuged for 20 min at 4 °C and 12 000 × *g*, and a total volume of 50 μL of supernatant was added into an HPLC vial.

L-Tyrosine hydroxylation by SrTH *in vitro*

After verification by DNA sequencing, the plasmids harboring SrTH were transferred by electroporation into *B. licheniformis*. The positive recombinants were first selected by the phenotype showing kanamycin resistance and further confirmed by PCR. *B. licheniformis* transformants were inoculated into 50 mL of fermentation medium supplied with 30 μg mL^{-1} kanamycin and 3% (v/v) seed cultures in 250 mL flasks and cultured at 37 °C and 250 rpm for 24 h. After fermentation, the OD_{600} was measured. The cells were collected by centrifugation at 4 °C and 12 000 rpm for 10 min, washed twice with 10 mmol L^{-1} sodium phosphate buffer (pH 7.0), and resuspended in the above buffer to an OD_{600} of 5. After treatment with 5 g L^{-1} lysate in a 37 °C incubator for 30 min, the cell suspension was ultrasonically broken and centrifuged at 4 °C and 12 000 rpm for 20 min to obtain the crude enzyme solution. The crude enzyme solution was frozen at −70 °C for 12 h and lyophilized into powder using a Benchtop Pro freeze-dryer (SP Scientific, New York, USA). The protein content was quantified by the Bradford assay[29] using bovine serum albumin (BSA) as a standard. The gene product of SrTH was confirmed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel as described by U. K. Laemmli.[30] Protein bands were stained using a protein stains Q kit (Sangon Biotech, Shanghai, China).

In the reaction, we supplemented with 1.7 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid and 100 μmol L^{-1} Fe^{2+} one time. At the same time, 1 g L^{-1} crude enzyme powder containing SrTH and 0.25 mmol L^{-1} BH4 were supplemented in the solution per 1 hour. After catalyst at 37 °C 180 rpm for 6 h, L-Dopa in the supernatant was detected by HPLC.

Analysis of the interaction between SrTH and pterins by the Autodock 4.0 program

The sequence identity of complete sequence and protein functional area is 22.29% and 38.02% between SrTH and HuTH (PDB ID: 2xsn), which analyzed by searching for templates in SWISS-MODEL.[31] The SrTH model was built by SWISS-MODEL[31] with HuTH as template and structurally evaluated by the SAVES program (https://servicesn.mbi.ucla.edu/SAVES/) (Fig. S1†), which runs PROCHECK, Verify3D, What_Check, and ERRAT at the same time.[32] The PDB structures of BH4 and dihydrobiopterin were retrieved from PubChem Compound of the NCBI GenBank database. Protein–ligand interaction studies between SrTH and pterins were carried out using Discovery Studio 3.0 (Accelrys) and Autodock 4.0 programs.

Product inhibition analysis of SrTH by dihydrobiopterin

In this study, we supplemented 1.5 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid, 100 μmol L^{-1} Fe^{2+}, 0.25 mmol L^{-1} BH4 per hour and 1 g L^{-1} crude enzyme powder containing SrTH per hour in the enzymatic process. During the progress of L-tyrosine hydroxylation by SrTH, we supplemented with 0.64 mmol L^{-1} 6,7-dihydrobiopterin or 1 mmol L^{-1} 7,8-dihydrobiopterin in the solution for the characterization of inhibition of dihydrobiopterin on hydroxylase. After catalyst at 37 °C 180 rpm for 3 h, L-Dopa in the supernatant was detected by HPLC.

Protein purification *via* DEAE-cellulose ion exchange chromatography

The mobile phase was filtered through a 0.22 μm-pore-size membrane and degassed for 20 min before use. A DEAE-cellulose column (column volume 5 mL) was pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) for 20 min.[49] The protein sample was linearly eluted with sodium phosphate buffer containing NaCl from 0–1 mol L^{-1} at a flow rate of 2 ml min^{-1}. Fractions were collected every 2 min. The absorbance of these fractions was measured at 280 nm. The protein content was quantified by the Bradford assay[29] using bovine serum albumin (BSA) as a standard.

Fluorescence measurements of pterin-SrTH systems

The protein concentration was 44 mg L^{-1}. The concentration of BH4/BH2/BH2 with adding 10 μmol L^{-1} BH4/PH2 were prepared from 0 to 20 μmol L^{-1} for SrTH. The fluorescence scanning was achieved in the range of 300–450 nm with 265 nm of excitation wavelength at four temperatures (298, 303, 310 and 315 K) according to the previous study.[45]

Characterization of inhibition by BH2 on SrTH

Kinetic studies on the interaction between tyrosine hydroxylase and L-tyrosine, oxygen and tetrahydropteridines were previously performed.[20,21,33] In this study, we supplemented 1.5 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid, 100

μmol L^{-1} Fe^{2+} and 20 mg L^{-1} SrTH purified *via* ion exchange chromatography in the enzymatic process. At the same time, 0.05–0.25 mmol L^{-1} BH4 and 0–0.4 mmol L^{-1} 7,8-dihydrobiopterin were supplemented in the enzyme reaction. After 1 h at 37 °C, the reaction was terminated by a final concentration of 5% trichloroacetic acid, and the production of L-Dopa was detected by HPLC. The kinetic parameters K_m and V_{max} were analyzed by a Lineweaver–Burk plot (double reciprocal plot), which utilizes 1/[S] as the *X* axis and 1/*V* as the *Y* axis (1).

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

K_i value was analyzed by a Lineweaver–Burk plot (double reciprocal plot), which utilizes 1/[S] as the *X* axis and 1/*V* as the *Y* axis (2).

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

where *V* is the rate of the reaction, V_{max} is the maximum rate of reaction, K_m is the Michaelis–Menten constant and indicates the affinity of the substrate to the enzyme, K_i is the inhibition constant of inhibitor, [S] is the substrate concentration and [I] is the inhibitor concentration.

Analysis of L-Dopa, BH4 and dihydrobiopterin

The samples were treated with a final concentration of 5% trichloroacetic acid. After incubation for 12 h at 4 °C, the samples were centrifuged for 20 min at 4 °C and 12 000 × *g*, and a total volume of 50 μL of supernatant was transferred to an HPLC vial. The supernatant was analyzed by a Thermo HPLC UltiMate 3000 comprising a quaternary pump, an autosampler, and a UV detector. Samples were separated on a Waters XSelect®HSS T3 C18 column. The mobile phase for L-Dopa analysis consisted of 97% 10 mmol L^{-1} sodium acetate (HPLC) and 3% acetonitrile (HPLC). The mobile phase for BH4 and dihydrobiopterin detection consisted of 97% 30 mmol L^{-1} sodium acetate (HPLC) and 3% methanol (HPLC).[34] The mobile phase was filtered through a 0.22 μm-pore-size membrane and degassed for 20 min before use. The column temperature was maintained at 40 °C, while detection was monitored at wavelengths of 280 nm for L-Dopa and 246 nm for BH4 and dihydrobiopterin. The injection volume of the samples was set at 10 μL with a flow rate of 1 mL min^{-1}. A standard curve was constructed from serial dilutions of a standard stock solution.

Results

Computer-assisted sequence analysis of tyrosine hydroxylases

The conserved amino acid positions and substrate binding site of tyrosine hydroxylase from animals have been elaborated.[21,35,39] Forty-one sequences of TH from different species were obtained from the NCBI GenBank database, which belonged to Actinobacteria, flatworms, nematodes, mollusks, arthropods, green plants, fungi and chordates. The sequences were used to construct a maximum-likelihood phylogenetic tree by the PhyML program. Finally, we screened out 7 tyrosine hydroxylases from Actinobacteria with similar conservative amino acid positions as shown in Fig. 1. The accession numbers for these sequences are shown in the figure. Analysis of amino acid sequences indicated that tyrosine hydroxylase from Actinobacteria is the earliest in evolutionary time and has the farthest relationship with chordates (Fig. 1A).

To analyze the conserved amino acid positions of tyrosine hydroxylase from different species during the evolution process, multiple sequence alignment (MSA) with secondary structure depiction (HuTH as template) was performed on these sequences through MAFFT and ESPript (Fig. 1B). We found that the consensus of 20 amino acid positions was 100% in all species, including Pro270, Gly284, Phe285, Phe299, Phe308, Arg315, Pro320, Pro326, Asp327, His330, Gly334, His335, Gly352, Trp371, Glu375, Gly377, Gly389, Ala390, Ser394, and Glu398. Notably, some important amino acid positions were not absolutely conserved, such as Gly292, Leu293, Leu294 and Tyr370, which are important for pterin binding. For example, tyrosine hydroxylase (PDB ID: EFE75625.1) had a G292A amino acid substitution in *Streptomyces filamentosus* NRRL15998, which may damage flexibility in this loop region and affect pterin binding. Similarly, the tyrosine hydroxylase (PDB ID: KPI05405.1) from *Actinobacteria bacterium* OK006 revealed L293T amino acid substitutions, which may influence the formation of hydrogen bonds from C-1′OH of pterin.[35] The tyrosine hydroxylases (PDB ID: KPI20637.1, EGD43416.1, ANZ14715.1, AJC54316.1 and ACY06282.1) also revealed L293I, L293I, L293V, L293V and L293V amino acid substitutions in *Actinobacteria bacterium* OV450, *Nocardioidaceae bacterium* Broad-1, *Streptomyces noursei* ATCC 11455, *Streptomyces sp.* 769 and *Streptomyces flaveolus*, respectively.

In this study, we chose 7 tyrosine hydroxylases, namely, KPI05405.1, ACQ81820.1, ABW14799.1, CCH30170.1, ACZ90985.1, ADG87750.1 and ADG87750.1, which were from *Actinobacteria bacterium* OK006, *Beutenbergia cavernae* DSM 12333, *Frankia sp.* EAN1pec, *Saccharothrix espanaensis* DSM 44229, *Streptosporangium roseum* DSM 43021, *Thermobispora bispora* DSM 43833 and *Thermomonospora curvata* DSM 43183, respectively, for further research. The synthesized sequences and GenBank accession numbers of 7 tyrosine hydroxylases are listed in Table S3.†

Functional identification of TH/SPR dual-enzyme synthesis systems

Preliminary studies have shown that it requires the participation of BH4, a cofactor produced by the folate synthesis pathway, in the process of catalyzing L-Dopa synthesis by TH.[25] To synthesize BH4 in *B. licheniformis*, we screened 6 sepiapterin reductases from microorganisms according to the tyrosine hydroxylase screening method. The

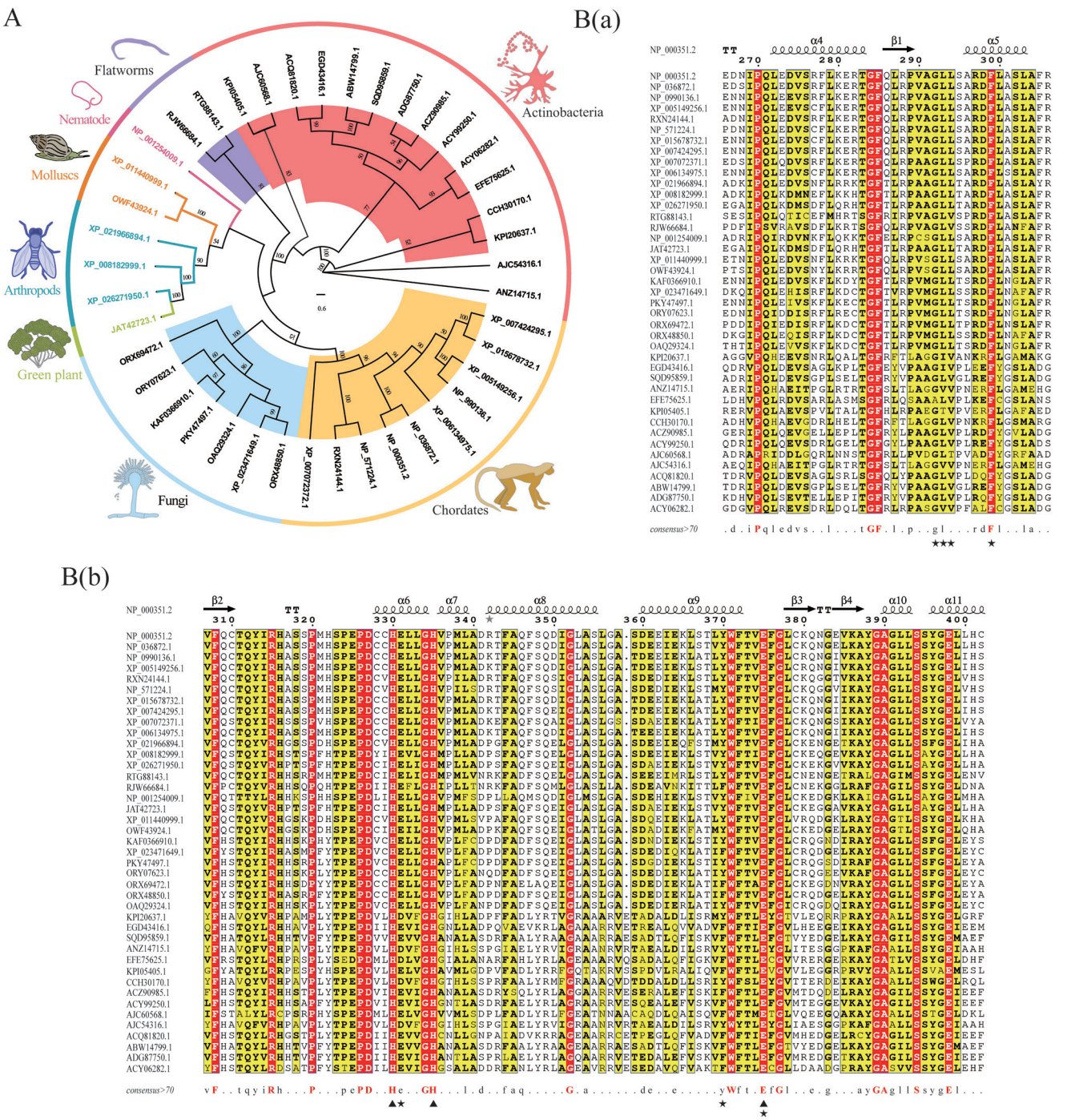


Fig. 1 Computer-assisted sequence analysis of tyrosine hydroxylases. A maximum-likelihood phylogenetic tree using amino acid sequences built using the PhyML software after selecting the best amino acid substitution model by ProtTest v3.4.2; B multiple sequence alignments of protein sequences harboring secondary structure depiction (HuTH as template) performed with the program MAFFT (MAFFT-linsi) v7.471 and visualized with ESPript 3.0. (a) amino acid positions of HuTH from 266 to 306; (b) amino acid positions of HuTH from 307 to 402.

synthesized sequences and GenBank accession numbers of 6 sepiapterin reductases are listed in Table S3.†

In order to quickly and efficiently screen out functional TH/SPR dual-enzyme synthesis systems, we use combined whole cell to catalyse L-tyrosine for producing L-Dopa. The resting cells of tyrosine hydroxylases were combined pairwise with that of sepiapterin reductases for L-Dopa production at 37 °C 250 rpm for 36 h (Fig. 2). During the catalytic process, 2 g L^{-1} L-tyrosine was added into the reaction solution as the substrate for L-Dopa. Obviously, the color of some combination solutions changed to black after whole cell transformation, indicating that L-Dopa was successfully synthesized in the catalytic process. L-Dopa will be oxidized to dopaquinone in the air and eventually become melanin. We detected L-Dopa of the 42

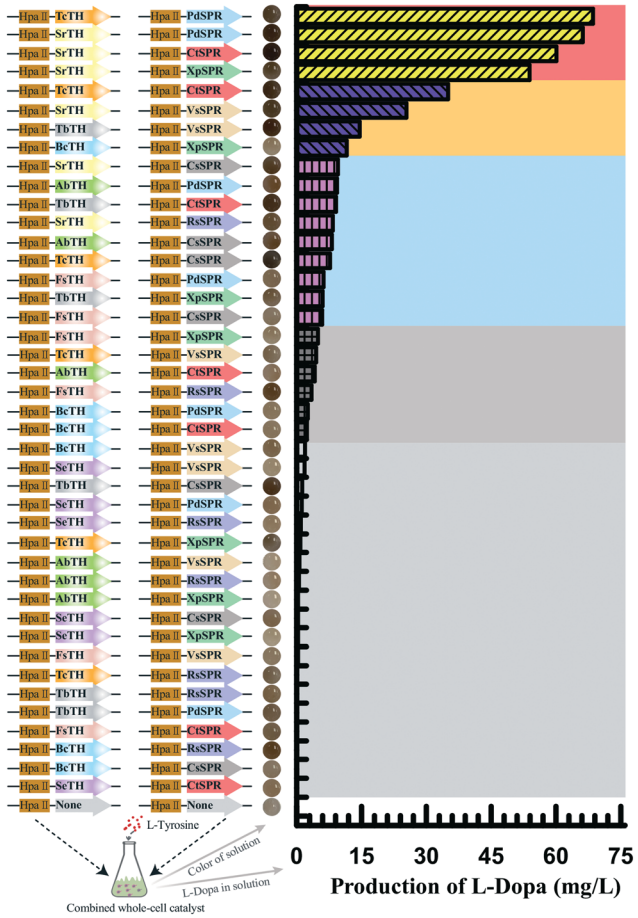


Fig. 2 Functional identification of TH/SPR dual-enzyme synthesis systems by combined whole-cell catalyst. The two kinds of cells expressing TH or SPR were mixed by OD_{600} 10 : OD_{600} 10 in two-by-two combination with 10 mmol L^{-1} sodium phosphate buffer (pH 7.0). In the process, 2 g L^{-1} L-tyrosine was added and the reaction was at 37 °C, 250 rpm for 24–36 h with 30 mL in the 250 mL flasks. After combined whole-cell catalyst by different groups, the color change was recorded and L-Dopa in the supernatant was detected by HPLC. All assays were performed in triplicate.

combination solutions by HPLC. The synthesis of L-Dopa was completely undetectable in 7 of 42 combinations, and the L-Dopa concentration of 18 combinations was less than 5 mg L^{-1}. We found that the combinations with high L-Dopa content basically focused on the combinations containing SrTH and TcTH. There were four combinations with L-Dopa concentrations above 50 mg L^{-1}, including TcTH/PdSPR (68.59 mg L^{-1}), SrTH/PdSPR (66.24 mg L^{-1}), SrTH/CtSPR (60.11 mg L^{-1}), and SrTH/XpSPR (53.87 mg L^{-1}). According to the phylogenetic tree (Fig. 1A), SrTH displayed a close relationship to TcTH. Both of these proteins play a potential role in the production of L-Dopa. Although the results were novel for producing L-Dopa by TH, the production of L-Dopa by combined whole-cell catalyst was lower than that of the other reported L-Dopa synthesis systems, such as HpaBC and tyrosinase. To unravel other limiting factors in the enzyme reaction, we characterized the properties of L-tyrosine hydroxylation by TH in the enzymatic process.

Hydroxylation of L-tyrosine with external BH4 supplementation

BH4 is the limiting factor in the process of tyrosine hydroxylase catalysis.[24] In this study, we attempted to use SrTH to catalyze tyrosine for the synthesis of L-Dopa without external BH4 supplementation. However, L-Dopa cannot be detected, indicating that SrTH is also a kind of BH4-dependent tyrosine hydroxylase.

To identify other bottlenecks in the enzymatic process, we retained sufficient enzyme, L-tyrosine, ascorbic acid (Fig. S2†) and BH4 levels in the crude enzyme reaction. To ensure that SrTH can be continuously added, the resulting enzyme solution after cell disruption was centrifuged and lyophilized to obtain a powder. The enzyme powder was dissolved in ddH_2O and analyzed using SDS-PAGE. Fig. 3A shows that SrTH had been successfully expressed in *B. licheniformis*. In the enzymatic process, we supplemented the reaction with 1.7 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid and 100 μmol L^{-1} Fe^{2+} one time. At the same time, 1 g L^{-1} crude enzyme powder containing SrTH and 0.25 mmol L^{-1} BH4 was supplemented per hour. L-Dopa was continuously synthesized in the first three hours, and its content increased to 90 mg L^{-1} at 3 h. However, L-Dopa did not continue to accumulate after 3 h (Fig. 3B). At the same time, we prepared the crude enzyme powder of other 6 screened enzymes (TcTH, TbTH, FsTH, BcTH, AbTH and SeTH) according to the same method as before. Due to low catalytic efficiency of TbTH, FsTH, BcTH, AbTH and SeTH as shown in Fig. 2 and S17,† L-Dopa synthesized *in vitro* by crude enzyme powder could not reach the limit of detection of HPLC. Only TcTH can synthesize 37.39 mg L^{-1} L-Dopa in the catalysis process as shown in Fig. S3,† which was much lower than that by SrTH. We analyzed the substances present in the reaction found that when BH4 was continuously added, dihydrobiopterin accumulated continuously, which may be the reason why L-Dopa did not accumulate continuously (Fig. S4†).

Subsequently, Autodock 4.0 was used to research the protein–ligand interactions between SrTH and BH4, 6,7-dihydrobiopterin or 7,8-dihydrobiopterin. We found that the other two substances interacted with the active center of binding BH4 in SrTH. Glu163 of SrTH can simultaneously form hydrogen bonds with BH4, 6,7-dihydrobiopterin and 7,8-dihydrobiopterin (Fig. 4). In particular, BH4 and 7,8-dihydrobiopterin have four common amino acid sites for interaction, namely, Leu125, Phe131, His167 and Glu163 (Fig. 4C). The docking results further indicate that the accumulation of BH4 oxidation products is likely to inhibit the synthesis of L-Dopa in the enzyme reaction.

Inhibition of enzyme reaction by dihydrobiopterin

BH4 is extremely unstable and will be oxidized to 6,7-dihydrobiopterin, which can be naturally converted to 7,8-dihydrobiopterin (Scheme 1).[34,36] The oxidation process of BH4 at room temperature was analyzed by HPLC. We found that the oxidation product of BH4 was a mixture of 6,7-dihydrobiopterin and 7,8-dihydrobiopterin during the

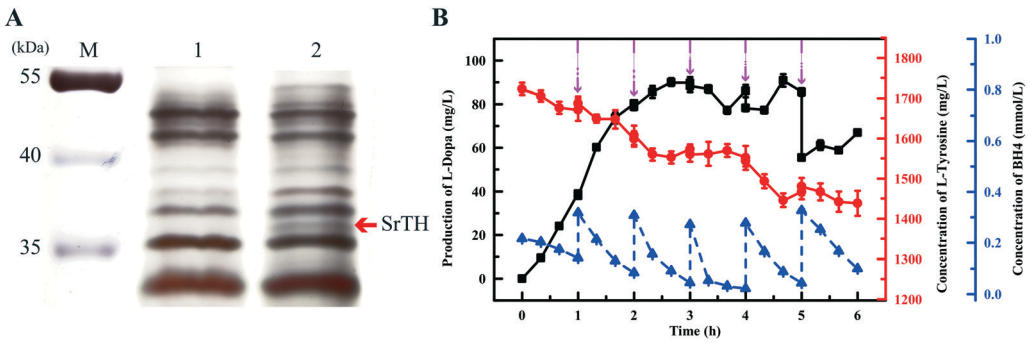


Fig. 3 Hydroxylation of L-tyrosine with external BH4 supplementation. A SDS-PAGE analysis of crude enzyme powder containing SrTH. Protein content was quantified by the Bradford assay using bovine serum albumin (BSA) as standard. 10 μg protein of (M) molecular weight marker, (lane 1) *Bacillus licheniformis* (blank) and (lane 2) *Bacillus licheniformis* (SrTH) was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel. Protein bands were stained by silver staining using a Protein Stains Q kit; B production of L-Dopa by crude enzyme powder containing SrTH. In the reaction, we supplemented with 1.7 g L^{-1} tyrosine (●), 5 g L^{-1} ascorbic acid and 100 μmol L^{-1} Fe^{2+} at a time. At the same time, 1 g L^{-1} crude enzyme powder containing SrTH and 0.25 mmol L^{-1} BH4 (▲) were supplemented in the solution per 1 hour. After catalyst at 37 °C 180 rpm for 6 h, L-Dopa (■) in the supernatant was detected by HPLC. The dash lines indicate the feed points.

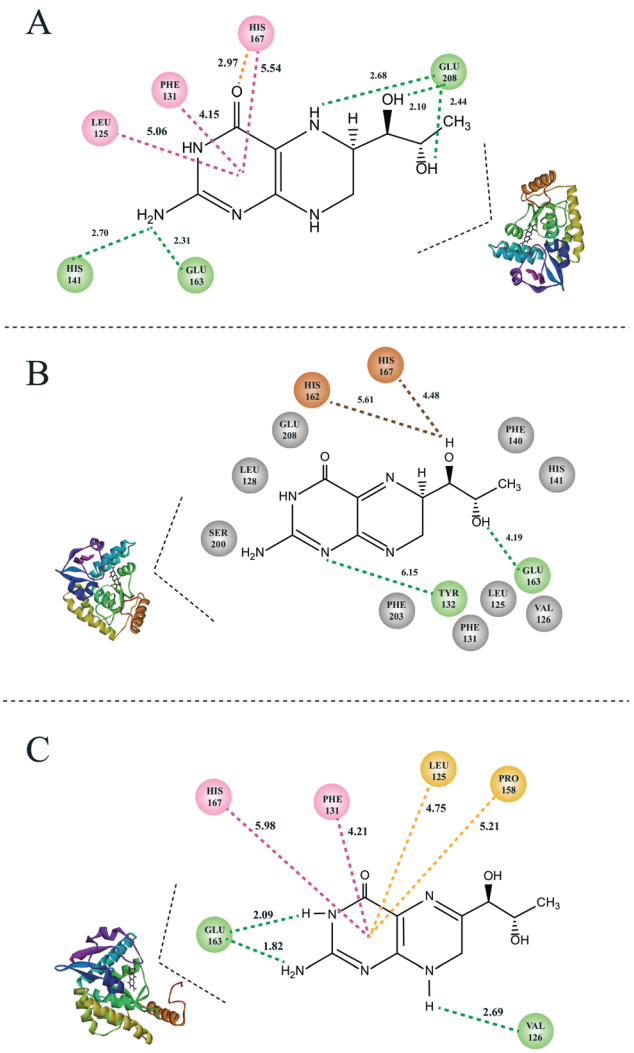
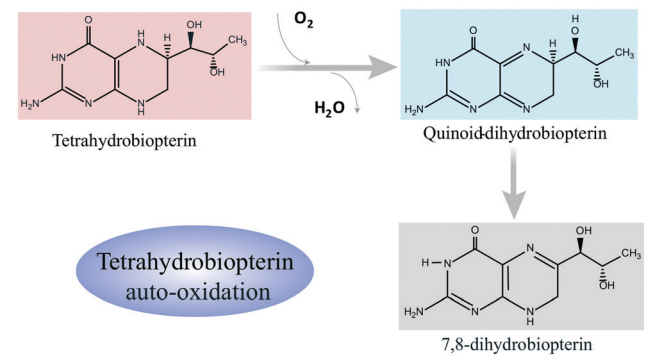


Fig. 4 Analysis of interaction between SrTH and pterins. The protein–ligand interactions between SrTH and BH4, 6,7-dihydrobiopterin or 7,8-dihydrobiopterin were studied using Discovery Studio 3.0 and Autodock 4.0. A Tetrahydrobiopterin (BH4); B 6,7-dihydrobiopterin (q-BH2); C: 7,8-dihydrobiopterin (BH2).

reaction and was finally 7,8-dihydrobiopterin at the end of the process. It was difficult to obtain high-purity 6,7-dihydrobiopterin during the oxidation of BH4 (Fig. S5 and S6†). This goal was still not achieved when increasing the temperature and the dissolved oxygen content during oxidation, and only 0.32 mmol L^{-1} 6,7-dihydrobiopterin could be obtained after incubation at 37 °C 250 rpm for 24 h (Fig. 5A). BH4 was converted to 7,8-dihydrobiopterin at 60 h (Fig. S7†). To study the influence of 6,7-dihydrobiopterin on the enzyme reaction, we tried to prepare high-purity 6,7-dihydrobiopterin using redox agents. 2,6-Dichloroindophenol is a redox agent that can be used to detect ascorbic acid and quickly oxidize BH4 to 6,7-dihydrobiopterin.[37] In this study, equimolar BH4 and 2,6-dichloroindophenol were mixed uniformly at 37 °C and 250 rpm. The oxidation products were detected by HPLC. We found that 6,7-dihydrobiopterin could accumulate to 0.64 mmol L^{-1} at 1.3 h, a higher concentration than that obtained without 2,6-dichloroindophenol (Fig. 5B and S8†).

Subsequently, 1 mmol L^{-1} 7,8-dihydrobiopterin and 0.64 mmol L^{-1} 6,7-dihydrobiopterin were added to the solution during the crude enzyme reaction, and the synthesis of L-Dopa was analyzed by HPLC (Fig. 6). We found that the



Scheme 1 The reaction path of BH4 auto-oxidation.

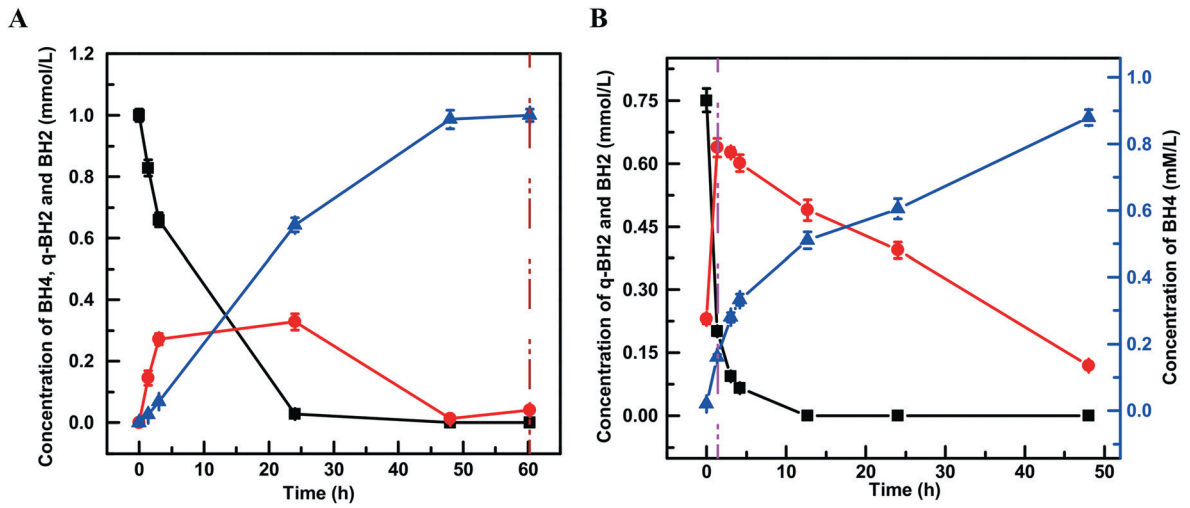


Fig. 5 Analysis of BH4 oxidation. A BH4 oxidation at natural condition. 1 mmol L^{-1} BH4 was shaked at 37 °C 250 rpm and the chemicals BH4 (■), BH2 (▲), q-BH2 (●) were detected by HPLC. The dash line indicates the point for preparing 7,8-dihydrobiopterin (BH2); B BH4 oxidation by 2,6-dichloroindophenol. The solution containing 1 mmol L^{-1} BH4 and 2,6-dichloroindophenol was shaked at 37 °C 250 rpm and the chemicals BH4 (■), BH2 (▲), q-BH2 (●) were detected by HPLC the dash line indicates the point for preparing 6,7-dihydrobiopterin (q-BH2).

synthesis of L-Dopa was not affected by the addition of 0.64 mmol L^{-1} 6,7-dihydrobiopterin (Fig. 6A). It is worth noting that when 1 mmol L^{-1} 7,8-dihydrobiopterin was added, the production of L-Dopa was reduced by 57%, decreasing from 81 mg L^{-1} to 35 mg L^{-1} (Fig. 6B). This indicates that the accumulation of 7,8-dihydrobiopterin can inhibit the synthesis of L-Dopa during the progress of the enzyme reaction, which is consistent with our previous speculation. We also evaluated the effect of 7,8-dihydrobiopterin on TcTH. It is worth noting that when 1 mmol L^{-1} 7,8-dihydrobiopterin was added, the production of L-Dopa by TcTH was reduced by 34%, decreasing from 37.39 mg L^{-1} to 24.79 mg L^{-1}, which was lower than 57% of SrTH (Fig. S3†). This indicates that the accumulation of 7,8-dihydrobiopterin can also inhibit the synthesis of L-Dopa during the progress of the enzyme reaction by TcTH, which is consistent with that by SrTH.

Characterization of inhibition by BH2 on SrTH

To obtain pure protein, we tried to express all the genes using pET28a in the *E. coli* expression system. Unfortunately, we could not be able to obtain the active protein and a large number of inclusion bodies would be formed during the expression process as shown in Fig. S9.† In order to analyze the characterization of SrTH, the crude enzyme was partially purified *via* ion exchange chromatography. The specific enzyme activity of SrTH reached 144.80 nmol min^{-1} (mg of protein)$^{-1}$ at 37 °C in this study as shown in Table S4 and Fig. S10.† In this study, we immediately determined the optimal catalytic temperature and pH of SrTH. We found that over 50% specific enzyme activity of SrTH can be detected at pH from 5 to 7.4 and at temperature from 20 °C and 70 °C as shown in Fig. S11,† which indicates that SrTH has better catalytic ability at a wider temperature range than that from animals.[20,40]

It was previously reported that hTH1 would be competitively inhibited by some BH4 analogs in the reaction, such as 4-amino-BH4 and 5-methyl-BH4 (Table 2), whose substituents were –NH2 and –CH3, respectively.[20] There is no report on 7,8-dihydrobiopterin inhibiting SrTH until now. The binding constant (K) for BH4/BH2/BH2 with adding 10 μmol L^{-1} BH4/PH2 and SrTH interaction at different temperatures were obtained by analyzing the fluorescence changes data recorded at 265 nm according to the previous study.[45] As shown in the illustrations of Fig. S12–S15,† the plot of $1/(F_0 - F)$ against 1/[Q] was observed to be linear, confirming a one-to-one interaction between the two partners. The thermodynamics parameters were also calculated by the binding constants between BH4/BH2/BH2 with adding 10 μmol L^{-1} BH4/PH2 and SrTH systems at different temperatures as shown in Table 1 and Fig. S12–S15.† Thermodynamic parameters including the free energy change (ΔG), the enthalpy change (ΔH) and the entropy change (ΔS) for the interaction between BH4/BH2/BH2 with adding 10 μmol L^{-1} BH4/PH2 and SrTH are vital for characterizing the binding forces involved in the BH4/BH2/BH2 with adding 10 μmol L^{-1} BH4/PH2–protein complex formation. ΔH and ΔS are positive indicating that the hydrophobic interaction is dominating acting forces in BH2/PH2–SrTH complex formation, which is different from the interactions between BH4 and SrTH.[46] The negative enthalpy (ΔH) and entropy (ΔS) values of the interaction of BH4 and SrTH indicate that van der Waals interactions and hydrogen bonds played major roles in the reaction.[46] It is worth noting that ΔH and ΔS of the interaction of BH2 and SrTH with adding 10 μmol L^{-1} BH4 in the solution changes into negative, which indicates that van der Waals interactions and hydrogen bonds are formed between BH2 and SrTH in the presence of BH4. This may due to that the binding of BH4 to SrTH can change the conformation of the protein and the

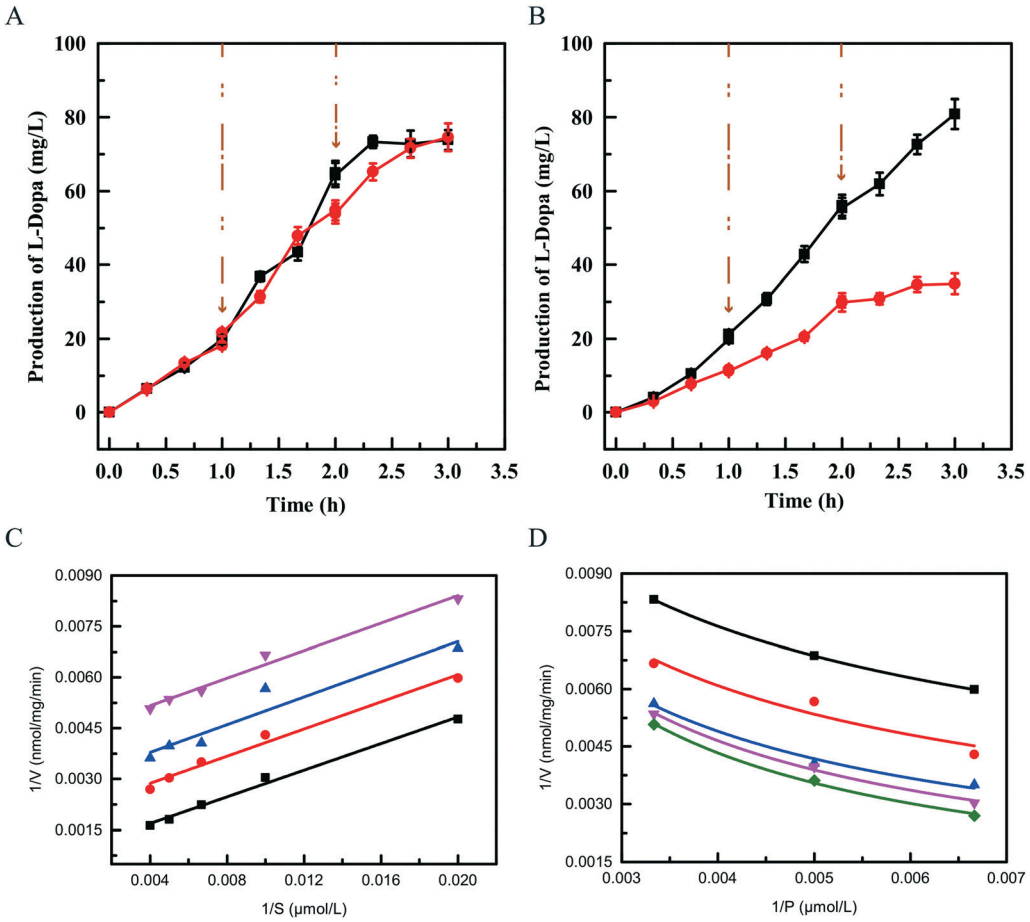


Fig. 6 Inhibition analysis of enzyme reaction by 6,7-dihydrobiopterin and 7,8-dihydrobiopterin. A L-Dopa production with adding 6,7-dihydrobiopterin. 0.64 mmol L^{-1} q-BH2 was added into the reaction (●) with only adding 1 mmol L^{-1} 2,6-dichloroindophenol as control (■); B L-Dopa production with adding 7,8-dihydrobiopterin. 1 mmol L^{-1} BH2 was added into the reaction (●) with adding equal volume of ddH_2O as control (■). C Double reciprocal plot with taking 1/[S] as *X* axis and 1/*V* as *Y* axis. The concentrations of 7,8-dihydrobiopterin were 0 (■), 0.15 (●), 0.2 (▲), and 0.3 mmol L^{-1} (▼); D double reciprocal plot with taking 1/[P] as *X* axis and 1/*V* as *Y* axis. The concentrations of BH4 were 0.05 (■), 0.1 (●), 0.15 (▲), 0.2 (▼), 0.25 mmol L^{-1} (◆). In the reaction, we supplemented with 1.5 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid, 100 μmol L^{-1} Fe^{2+} and 1 g L^{-1} crude enzyme powder containing SrTH. 1 g L^{-1} crude enzyme powder containing SrTH and 0.25 mmol L^{-1} BH4 were supplemented in the solution per 1 hour in A and B. 0.05–0.25 mmol L^{-1} BH4 and 0–0.3 mmol L^{-1} 7,8-dihydrobiopterin were supplemented in the enzyme reaction of C and D. After catalyst at 37 °C 180 rpm, the production of L-Dopa was detected by HPLC. The dash lines indicate the feed points. All assays were performed in triplicate.

Table 1 Binding parameters and thermodynamic parameters for the binding of various pterins to SrTH

System	*T* (K)	K_a (10^4 L mol^{-1})	R^a	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔH (kJ mol^{-1})	R^b
SrTH (BH4)	303	42.22	0.9929	−32.93	−53.97	−49.02	0.9994
	310	27.63	0.9966	−32.66			
	315	20.09	0.9957	−32.28			
SrTH (BH2)	298	18.53	0.9996	−30.04	315.48	64.01	0.9991
	303	27.59	0.9986	−31.62			
	310	50.19	0.9978	−33.83			
SrTH (BH4&BH2)[c]	303	33.15	0.9924	−35.01	−624.6	−221.2	0.9941
	310	3.710	0.9970	−31.88			
	315	1.205	0.9937	−27.51			
SrTH (PH2)	298	16.90	0.9950	−29.27	526.16	127.60	0.9968
	303	33.54	0.9963	−31.90			
	310	95.87	0.9907	−35.59			

[a] *R* is the correlation coefficient for the K_a values. [b] *R* is the correlation coefficient for the van't Hoff plot. [c] 10 μmol L^{-1} BH4 was added into the solution before detecting the binding parameter of BH2.

Table 2 Kinetic constants for tetrahydropterins in the reaction catalyzed by TH

Tetrahydropterin	Enzymes	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	Ref.
6(*R*)-BH4	hTH1	27.4 ± 6.3	1146 ± 330	20
6(*S*)-BH4	hTH1	218 ± 26	492 ± 7	20
6-MPH4	hTH1	35.9 ± 4.7	720 ± 79	20
6(*R*)-BH4	SrTH	224 ± 19	1108 ± 120	This study

The values represent the mean with the standard errors calculated from 3 experiments.

changed protein conformation can form van der Waals interactions and hydrogen bonds with BH2. In order to further explain the effect of hydrogen bonds between BH2 and SrTH, an appropriate amount of 7,8-dihydrobiopterin was added to the reaction to systematically analyze the influence of 7,8-dihydrobiopterin on the activity of SrTH. We found that the slope rate indicating K_m/V_{max} of the four straight lines in the double reciprocal plot is the same and was 0.19647, 0.20043, 0.20442 and 0.20268 after adding 0, 0.15, 0.2, and 0.3 mmol L^{-1} 7,8-dihydrobiopterin, respectively, into the reaction catalyzed by SrTH (Fig. 6C). The K_m and V_{max} values decreased, and K_m/V_{max} remained constant as the 7,8-dihydrobiopterin content increased (Fig. 6D), which indicates that the inhibitory effect of 7,8-dihydrobiopterin on SrTH is anticompetitive inhibition. In this study, we got the kinetic constants for tetrahydropterins in the reaction catalyzed by SrTH and K_i of 7,8-dihydrobiopterin on SrTH, which is 224 μM (K_m), 1108 nmol mg^{-1} min^{-1} (V_{max}), and 98 μM (K_i) as shown in Tables 2 and 3. It is worth noting that there is no obvious difference in K_i constant between crude SrTH and SrTH partially purified *via* ion exchange chromatography as shown in Table S5.†

Effect of pure EcDHPR addition on the reaction catalyzed by SrTH

The gene *nfsB*, encoding EcDHPR, was cloned into pET28a and expressed in Bl-21 (DE3) at 16 °C and 200 rpm for 24 h with the addition of 0.1 mmol L^{-1} IPTG. The product of the gene *nfsB* was analyzed by SDS-PAGE using 12% polyacrylamide gel, which indicated that the soluble expression of EcDHPR was successfully achieved (Fig. 7A). Subsequently, EcDHPR was purified using a HisTrap affinity column (Ni-NTA), and the pure protein had a yellow color, which is consistent with previous research (Fig. 7B).[38] We detected the synthesis of BH4 by HPLC using 0.1 g L^{-1} pure EcDHPR, 0.5 mmol L^{-1} q-BH2 and 1 mmol L^{-1} NADH at 25 °C for 1 h in HEPES-NaOH (pH 7.4) buffer (Fig. S16†). In the enzyme reaction, 1 mmo L^{-1} NADH was almost completely converted into NAD (+), and the content of BH4 produced by EcDHPR was 58.52 μmol L^{-1} in the solution at 1 h. In this study, we attempted to use BH4 regeneration by EcDHPR to alleviate BH2 inhibition of L-tyrosine hydroxylation by crude SrTH. During the reaction, we added 3 μg of EcDHPR and 1 mmol L^{-1} NADH per 1 h and found that L-Dopa could be produced continuously after 3 h and improved to 0.17 g L^{-1} at 6 h (Fig. 7C), which indicated that the BH4 regeneration pathway can alleviate the inhibition by BH2 during the reaction.

Discussion

To date, the tyrosine hydroxylases studied are derived from cows, rats and humans.[21,35,39] The actual titer of L-DOPA was not quantified, even though a new pathway had been demonstrated to hydroxylate L-tyrosine for producing L-Dopa by TH in *E. coli*. BH4 is replaced by tetrahydromonapterin (MH4) as the cofactor in a previous work.[25] In this study, we expressed tyrosine hydroxylases and sepiapterin reductases selected from microorganisms in *B. licheniformis* and successfully screened several TH/SPR synthesis systems that can hydroxylate L-tyrosine for synthesizing L-Dopa (Fig. 2), which provides a novel foundation for L-tyrosine hydroxylation by TH in the future. Simultaneously, we found anticompetitive inhibition of the SrTH-catalyzed reaction by 7,8-dihydrobiopterin, which has enriched theoretical support for subsequent research on the rational and semirational modification of SrTH and the biosynthesis of L-Dopa by the TH/SPR synthesis system in *B. licheniformis*.

L-Tyrosine hydroxylation by TH requires the participation of the cofactor BH4, which can be produced by GCHI, PTPS and SPR from GTP in the folate biosynthesis pathway (http://www.kegg.jp/kegg/). This reaction could generate 23 mg L^{-1}

Table 3 K_i values and type of inhibition for inhibitory or weakly binding pterins in the reaction catalyzed by TH

Pterins	Enzymes	K_i (μM)	Inhibition	References
4-Amino-BH4	hTH1	16	Competitive *vs.* 6(*R*)-BH4	20
5-Methyl-BH4	hTH1	63	Competitive *vs.* 6(*R*)-BH4	20
8-M-6,7-DMPH4	hTH1	617	Competitive *vs.* 6(*R*)-BH4	20
q-BH2	SrTH		No inhibition	This study
BH2	SrTH	98 ± 16	Anticompetitive *vs.* 6(*R*)-BH4	This study

The values represent the mean with the standard errors calculated from 3 experiments.

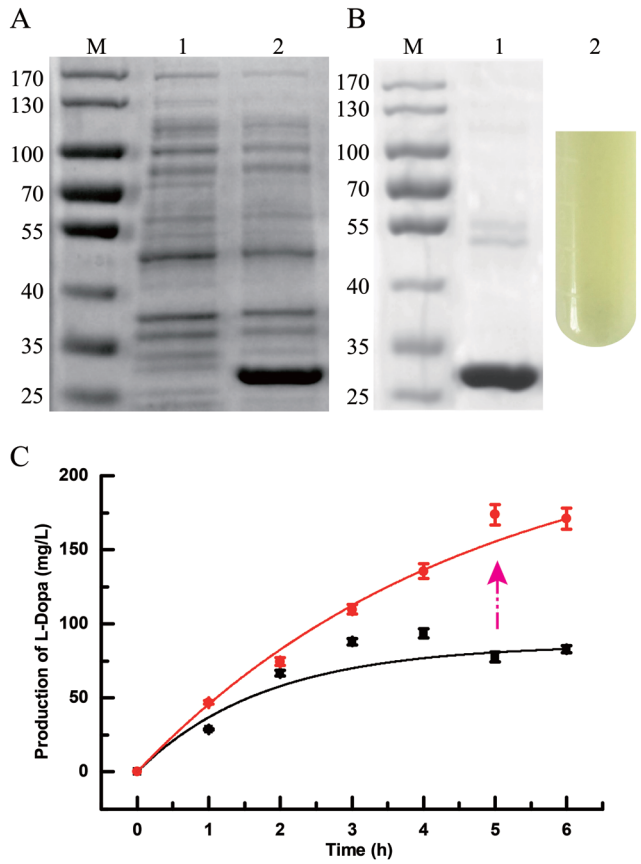


Fig. 7 Effect of pure EcDHPR addition on the reaction catalyzed by SrTH. Protein content was quantified by the Bradford assay using bovine serum albumin (BSA) as standard. 10 μg protein was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel. Protein bands were stained by Coomassie Brilliant Blue G250. A SDS-PAGE analysis of crude enzyme containing EcDHPR. (M) Molecular weight marker, (lane 1) *E. coli* Bl-21 (blank) and (lane 2) *E. coli* Bl-21 (EcDHPR); B SDS-PAGE analysis of pure enzyme EcDHPR. (M) Molecular weight marker, (lane 1) pure EcDHPR and (lane 2) color of EcDHPR; C production of L-Dopa with adding pure EcDHPR and NADH. In the reaction, we supplemented with 1.5 g L^{-1} tyrosine, 5 g L^{-1} ascorbic acid and 100 μmol L^{-1} Fe^{2+} at a time. At the same time, 1 g L^{-1} crude enzyme powder containing SrTH and 0.25 mmol L^{-1} BH4 were supplemented with per 1 hour. In the sample (●), we added 3 μg EcDHPR and 1 mmol L^{-1} NADH per 1 hour. And in the control (■), we added the same volume of HEPES-NaOH (pH 7.4). After catalyst at 37 °C 180 rpm for 6 h, L-Dopa in the supernatant was detected by HPLC.

BH4 when *folE* from *E. coli* and PTPS and SPR from rats were coexpressed in *E. coli*. Compared with *folE* from *E. coli*, the expression of GCHI encoded by the *mtrA* gene derived from *Bacillus subtilis* in *E. coli* can enhance the production of 5-hydroxytryptophan.[24] This was because when *folE* was substituted by GCHI from *B. subtilis*, BH4 productivity was improved 1.5-fold.[22] We analyzed the BH4 synthesis pathway of *B. licheniformis* by KEGG and found that it contained a complete synthetic pathway of the BH4 precursor 6-pyruvoyltetrahydropterin, in which GCHI has been proven to be functional.[22] Therefore, we proposed a speculation that *B. licheniformis* overexpressing exogenous SPR can synthesize BH4 in one step. The synthesis of L-Dopa indicates that BH4 can be successfully produced in *B. licheniformis* when PdSPR from *Photobacterium damselae* is overexpressed. However, we did not detect the production of BH4 in this article given that only SPR was expressed in *B. licheniformis*. Further metabolic engineering efforts are considered to analyze the synthesis of BH4 after increasing GTP synthesis and enhancing the expression of GCHI and PTPS in *B. licheniformis*, which will provide a novel foundation for the metabolic synthesis of L-Dopa in *B. licheniformis*.

The currently reported enzymes for L-tyrosine hydroxylation include HpaBC and tyrosinase.[13,18] There are excellent works on the production of L-Dopa by HpaBC, which can generate up to 0.69 g L^{-1} L-Dopa from glucose in shake flasks.[11,13,14] However, a large amount of melanin and tyrosine remaining in the fermentation broth causes loss of carbon flux to L-Dopa and its downstream high-value products, such as hydroxytyrosol, plant alkaloids, betalain pigments and monolignols.[4–8,25] Tyrosine hydroxylase does not have diphenolase (DP) activity, which is a good candidate to overcome this challenge.[25] Although the synthesis of L-Dopa was successfully achieved by a combined whole-cell catalyst in *B. licheniformis*, the titer of L-Dopa produced by L-tyrosine hydroxylation was lower than those of the other two enzymes in this study. There are four reasons that could explain this phenomenon. First, L-Dopa is easily oxidized to dopaquinone in the air and eventually becomes melanin (Fig. 2). Second, the production of BH4, an important cofactor during L-tyrosine hydroxylation by TH, is low in recombinant *B. licheniformis* expressing only PdSPR. During L-tryptophan hydroxylation to produce 5-hydroxytryptophan, both the synthesis and regeneration pathways of BH4 are strengthened, and the titer of 5-hydroxytryptophan can reach 1.61 g L^{-1}.[23,24] At the same time, BH4 is easily oxidized by O_2.[36] These results indicate that BH4 is a limiting factor during the hydroxylation of L-tyrosine by *B. licheniformis*. Unexpectedly, this system still cannot produce L-Dopa continuously and effectively when adding sufficient BH4 and crude SrTH protein during the catalysis process (Fig. 3B) because the hydroxylation of L-tyrosine by SrTH was inhibited by 7,8-dihydrobiopterin during the enzyme reaction (Fig. 6). We compared the K_i among the four different pterin compounds and found that inhibitory effect of BH2 (K_i 98 μM) on TH is weaker than 4-amino-BH4 (K_i 16 μM) and 5-methyl-BH4 (K_i 63 μM) but stronger than 8-M-6,7-DMPH4 (K_i 617 μM) as shown in Table 3. In this study, it was demonstrated that the BH4 regeneration pathway can not only provide more cofactor BH4 but also alleviate the anticompetitive inhibition of L-tyrosine hydroxylation by 7,8-dihydrobiopterin (Fig. 7C). Fourth, tyrosine hydroxylase can be affected by L-tyrosine (over 100 μM) and oxygen (higher than 4.8%) in the catalytic reaction.[21] Furthermore, SrTH lacks the N-terminal domain as a eukaryotic enzyme, which has the function of physically blocking the entrance to the active site in the substrate-free enzyme.[1] The reversible phosphorylation of serine residues in the N-terminal domain

plays an important role in decreasing the affinity for L-Dopa.[41,42] At the same time, the lack of an N-terminal domain may cause structural instability of the SrTH protein.[43] Finally, Leu295 and Tyr371 of rat tyrosine hydroxylase were important for forming hydrogen bonds with pterin, specifically with the N-8 and O-4 positions of pterin, respectively.[35] However, the two positions of SrTH were substituted by Val126 and Phe203, which may decrease the affinity for BH4. Next, site saturation mutagenesis will be performed on the Val126 and Phe203 amino acid positions of SrTH to research the influence on BH4 binding.

It will be a huge challenge to efficiently synthesize L-Dopa by hydroxylating L-tyrosine in *B. licheniformis* using the TH/SPR dual-enzyme synthesis system. In the future, we will focus on two research objectives regarding L-tyrosine hydroxylation by *B. licheniformis* harboring SrTH. Firstly, we will try to increase the expression of SrTH in *B. licheniformis*. Secondly, we will build a multigene pathway for producing L-Dopa and its high-value downstream products in *B. licheniformis* through a modular pathway engineering strategy based on previous research.[25,44,51]

Conclusions

In conclusion, we successfully built a TH/SPR dual-enzyme synthesis system and achieved L-tyrosine hydroxylation in *B. licheniformis* by functional SrTH and PdSPR. The synthesis of L-Dopa by L-tyrosine hydroxylation in *B. licheniformis* is restricted by many factors, including but not limited to tyrosine hydroxylase, BH4, L-tyrosine and oxygen. In the enzymatic catalysis process, only ensuring sufficient enzyme and substrate cannot continuously and efficiently generate L-Dopa. 7,8-Dihydrobiopterin formed by BH4 autooxidation is an anticompetitive inhibitor on tyrosine hydroxylase. Fortunately, the inhibition of L-tyrosine hydroxylation by 7,8-dihydrobiopterin can be alleviated through the BH4 regeneration pathway. Further studies should be considered to enhance the synthesis of BH4 on the one hand and build a BH4 regeneration pathway in *B. licheniformis* on the other hand. Our research provides a novel L-tyrosine hydroxylation system by utilizing a GRAS workhorse for food-grade L-Dopa production.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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